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The effects of LAIV on nasopharyngeal bacteria in healthy 2-4 year olds: a randomized controlled trial

Valtyr Thors, MD^{1,4}, Hannah Christensen, PhD², Begonia Morales-Aza¹, Ian Vipond, PhD³, Peter Muir, PhD³, Adam Finn, PhD¹

1: School of Cellular and Molecular Medicine, University of Bristol, Education Centre, Upper Maudlin Street, Bristol BS2 8AE, UK.

2: School of Social and Community Medicine, University of Bristol, Oakfield House, Oakfield Grove, Bristol, BS8 2BN.

3: Public Health Laboratory Bristol, Public Health England, Myrtle road, Bristol BS2 8EL, UK.

4: Present address: Pediatric Infectious Diseases & Immunology, Children's Hospital Reykjavik, Hringbraut 101, Reykjavik, Iceland.

Running head: Effects of LAIV on colonizing bacteria

Correspondence to:

Valtyr Thors

School of Cellular and Molecular Medicine

Education Centre, Upper Maudlin Street, Bristol BS2 8AE, UK

valtyr.thors@bristol.ac.uk

+44 (0)117 3420172

Contributions: AF and VT conceived of and designed the study. VT collected the samples. VT, IV and BMA performed the laboratory analysis. VT, HC, PM and IV analysed and interpreted the data. VT and AF drafted the manuscript. VT, HC, IV, BMA, PM and AF critically reviewed and edited the manuscript. VT and HC did the statistical analysis.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

At a glance commentary

Scientific knowledge on the subject:

Epidemiological data have suggested a link between respiratory viral and invasive and severe respiratory bacterial infections, while animal studies using wild and attenuated influenza strains have shown increases in density of *S. pneumoniae* and *S. aureus* in the nasopharyngeal mucosa. There is no experimental evidence on this subject in humans.

What this study adds to the field:

This randomized trial provides evidence that administration of live attenuated influenza vaccine to healthy children, after a short delay, increases the colonization density with *S. pneumoniae* in individuals carrying this bacterial species at the time of vaccination. It may also transiently increase rates of nasal colonization with *H. influenzae*. Comparison of pre- and post-vaccine samples by multivariate analysis confirms these findings and suggests that vaccination may also transiently increase the density of *M. catarrhalis* amongst carriers.

Abstract

Rationale Viral infections of the upper respiratory tract may influence the commensal nasopharyngeal bacteria. Changes in the bacterial niche could affect transmission dynamics. Attenuated vaccine viruses can be used to investigate this empirically in humans.

Methods We used trivalent Live Attenuated Influenza Vaccine (LAIV) to evaluate the effects of viral infection on bacterial carriage and density of *S.pneumoniae*, *M.catarrhalis*, *H.influenzae* and *S.aureus*. 151 healthy children were randomized 1:1 to receive the vaccine starting either at recruitment (n=74) or 28 days later (n=77) in a stepped wedge fashion, allowing comparisons between recipients and non-recipients as well as whole-group comparisons pre- and post- vaccination. Bacterial carriage and density were determined using qPCR assays.

Measurement & Main Results 151 children were recruited, 77 in the LAIV group and 74 controls. LAIV recipients (n=63 analysed) showed an apparent transient increase in *H.influenzae* carriage but no further significant differences in carriage prevalence of the four bacterial species compared to controls (n=72 analyzed). *S.pneumoniae* density was substantially higher in vaccine recipients (16687 vs 1935 GC/ml) 28 days after the first dose (p<0.001). Whole-group multivariable analysis (pre-vaccine, after one dose and after two doses) also showed increases in density of other species and *H. influenzae* carriage prevalence.

Conclusion In the absence of any safety signals despite widespread use of the vaccine, these findings suggest that bacterial density and thus, transmission rates amongst children and to other age groups may rise following attenuated influenza infections without associated clinical disease. LAIV could therefore be used as an experimental tool to elucidate the dynamics of transmission of nasopharyngeal bacteria.

Word count: 259

Key words: LAIV, Bacterial colonization, Children, Bacterial density

Introduction

Pneumococcal conjugate vaccines are effective principally through population-wide effects on carriage and transmission(1). Despite this, the biology of colonization and determinants of transmission remain poorly understood. Evidence from both animals(2) and humans(3) suggests that respiratory viral infections can affect upper respiratory tract (URT) bacteria but the relevance of experiments in animal models to humans is uncertain and deductions about causation are difficult from observational studies. Studies from our group and others have shown that presence and colonization density of commensal bacteria of the nasopharynx (NP) is associated with symptoms of rhinitis and presence of respiratory viruses(4, 5).

Live attenuated intranasal influenza vaccine (LAIV) was licensed in Europe in 2012 for children aged 2-18 years and until 2014 (when a second B strain was added) consisted of three replication competent viruses (two type A strains and one type B) attenuated by recombination of wild-type and attenuated donor viruses(6) and cold-adaptation through passages of progressively lower temperature in eggs(7). The vaccine viruses induce a broad cell mediated as well as local mucosal antibody responses(8) and replicate well at the lower temperatures of the upper respiratory mucosa but poorly in the lower airways or systemic circulation(9). LAIV is well tolerated, safe and effective in preventing culture/PCR proven influenza in children(10, 11) among whom flu is a common and sometimes serious infection. However, when compared to trivalent inactivated influenza vaccine (TIV), higher rates of medically significant wheezing were observed during the second to fourth weeks post-vaccination in children 6-23 months of age after 1st dose of vaccine (5.9% in LAIV recipients vs 3.8% in TIV recipients as shown in figure 3 in supplemental material from Belshe et al(12)). In addition, increased all-cause hospitalisation in children aged 6-11 months within 180 days of vaccination (6.1% in LAIV recipients vs 2.6% in

TIV recipients)(11), was observed, leading to restriction of licensure to children aged two years and older(13, 14). The pathophysiology of these adverse events is not understood.

Mina *et al.* found a transient increase in density and delayed clearance of NP *Streptococcus pneumoniae* and *Staphylococcus aureus* in a mouse model, after administration of either a single, cold-adapted live attenuated influenza virus strain or wild-type (WT) influenza virus. No clinical disease was seen in animals given attenuated virus although up to 5% weight loss was observed if *S. pneumoniae* was present before vaccination(15). Thus both WT and attenuated influenza infection could influence bacterial load in the human NP although extrapolation to humans and LAIV should be done with caution.

Demonstration of a similar effect in humans might help elucidate the relationship between respiratory viral infections and carriage prevalence and density dynamics of NP bacteria. The availability of LAIV which induces a mild URT infection without significant clinical illness offers an opportunity to undertake randomized interventional experimental studies in humans to explore this.

Real-time quantitative PCR (qPCR) can enumerate bacterial species-specific gene copies and thus both viable and non-viable bacteria in biological samples over wide concentration ranges with less effort and expense than conventional quantitative plate cultures. Using qPCR, we recently described upper respiratory bacterial density ranges as wide as 6 orders of magnitude in healthy children(16). Such differences may result in wide variation in efficiency of onward transmission amongst carriers. Although alternatives exist, nasopharyngeal swabbing is the most widely used method to sample the nasal flora, is easy to perform and reasonably well accepted by children.

To explore the effect of LAIV on carriage rates and density of four bacterial species which commonly colonize the human NP, we recruited 151 children to a prospective randomized study comparing vaccinated and unvaccinated children at one and four weeks post-vaccine and colonization before and after vaccination in the whole group. At the time of the study, no routine influenza immunization was being offered to healthy children in the UK.

Some of the results of this study have been previously reported in the form of an abstract(17).

Methods

Study design

This was a cohort stepped wedge study of a Live Attenuated Influenza Vaccine (LAIV) containing three (two A and one B) influenza strains, in children undertaken in 10 day care centers (DCC) in Bristol UK.

Participants and randomization

Children aged 2-4 years attending the DCC were eligible to participate. We attempted to provide written information sheets with an invitation to take part in the study to parents of all of the 659 eligible children in the ten DCCs; we were able to make contact with 80% of the parents and invite them to take part in the study between September 3 and October 15 2012. Only children for whom the vaccine was contraindicated were excluded (figure 1). Informed consent was obtained for all enrolled subjects. On enrolment, children were individually randomized [1:1] within nurseries using the BRTC electronic randomization system (Bristol Randomised Trials Collaboration, School of Social and Community Medicine, University of Bristol) by the study team, to receive the vaccine at day 0 (visit (V) 1) and on or around day 28

(V3) (LAIV group) or on or around day 28 (V3) and day 56 (V5) (control/ late vaccine group) with additional swabbing visits (V2 & V4) on or around days 7 and 35, respectively. A small number of children who had received inactivated influenza vaccine in either of the previous two seasons received only the first LAIV dose in the study (figure 1). If the child refused vaccination, NP swabbing only was attempted. Laboratory staff were blinded to the intervention assignment during analysis.

Intervention and procedures

Vaccine doses were given intra-nasally following monitored cold chain storage and after medical assessment of each subject. Following immunization, children were observed for 30 minutes. At all study visits, which were conducted at the DCCs between October 2012 and February 2013, a fine tip pediatric NP swab (Peel Pouch Dryswab™ Medical Wire & Equipment, Wiltshire, UK) was taken. For the swabbing procedure, the child was seated comfortably with the head slightly extended. The swab was inserted horizontally until resistance was met when the swab was retracted with a slight twisting action along the axis of the shaft and inserted into a 2ml tube prefilled with 1.5ml of Skim milk-Tryptone-Glucose-Glycerol (STGG) broth which was labelled with a random number to blind laboratory analysis. The records, linking the study participant and visit numbers with the blinding numbers were kept in a locked facility until after the completion of laboratory analysis. Tubes were held at 0-4°C until transferred to storage at -80°C within 4 hours of collection. At the time of collection of each swab, severity of any rhinitis symptoms was recorded ("SNOT score" 0-3 as previously reported(4) and described in the online supplement).

Laboratory analysis

Nucleic acid was extracted from all samples using the Virus/Pathogen mini kit® from QIAGEN (CA, USA) yielding 110µl extracts from 300µl STGG broth. These were stored at -80°C. Monoplex qPCR assays were used to detect *S. pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *S. aureus*(16). The cycle threshold (Ct) value to define detection was set at ≤35 cycles. For density values, Ct values were converted into gene copies (GC)/ml using standard curves(16). qPCR assays for adenovirus, bocavirus, influenza (A and B), respiratory syncytial virus, rhinovirus, human metapneumovirus, parainfluenza (type 1-3), parechovirus, coronavirus (229E, OC43, NL63) and enterovirus were performed using methods described previously(4, 18). In addition, an assay for coronavirus HKU1 (online supplement, table E1) was used.

Outcomes

The primary endpoint was a comparison of the proportion of children positive for *S. pneumoniae* one week after a first dose of LAIV compared with the control group. Additional planned endpoints included similar comparisons for *M. catarrhalis*, *H. influenzae* or *S. aureus* at one and four weeks after LAIV, of the density of all four species after the same intervals, both singly and combined and comparisons of the proportions of subjects positive and of carriage density for the four species before and after one or two doses of LAIV over all study visits both in all children studied and in those who either were or were not carriers of each species at the first visit. Exploratory and descriptive analyses, respectively, were also done with respect to rhinitis symptom scores and presence of respiratory viral nucleic acid in samples.

Statistical analysis

Statistical analyses were done using Stata® version 12.0 (StataCorp, College Station, Texas). To assess balance at randomization, differences characteristics were analyzed using Pearson's χ^2 test and t-tests when appropriate. We estimated the odds ratio (ORs) of carriage for each bacterial species in the early vaccine group compared to the control (late vaccine) group at V2 and V3 post enrolment using logistic regression, including a fixed effect for vaccine group and a random effect (intercept) for nurseries, to allow for between-nursery variability. We also estimated the ORs of carriage after one and two doses of LAIV (i.e. children contributed measurements before and after their vaccination) using logistic regression adjusting for confounders (detailed in footnotes to tables 4 and 5), with random effects (intercept) for nurseries and children to allow for repeated measures on the same individuals and between-nursery variability. We estimated the difference in mean density (GC/ml) of bacteria in those children positive for carriage (at baseline and the visit of interest) using gamma regression (because GC/ml were positively skewed), including the same fixed and random effects as for the odds of carriage analysis. We modelled the effect of the vaccine from one visit after the vaccine was administered. Analyses were done on the modified intention-to-treat (MITT) population, which included all enrolled participants who provided an assessable swab sample irrespective of whether they actually received the vaccine doses. Analyses were undertaken using *gllamm* program in Stata(19-21).

Trial registration

Trial registration was initiated (Eudract nr: 2011-002964-25) but owing to regulatory authority determination that this was not an IMP trial, since the vaccine was licensed for this age group, could not be completed.

Role of the funding source

The funding bodies for the study had no role in the study design, data collection, data analysis, data interpretation or writing or approval of the manuscript. All authors had full access to all the data in the study and the corresponding author had responsibility for submitting the manuscript for publication.

Ethical approval

The study was approved by the UK National Research Ethics Service – Central Bristol Committee (ref nr. 11/SW/0186) and responsibility for governance oversight taken by the University of Bristol, UK.

Results

A consort diagram detailing screening, enrolment, randomization, loss to follow up and completion is shown in figure 1 (see details by nursery in online supplement table E2). A total of 125 children completed the vaccination protocol and 120 completed all visits (six withdrew before or at the time of the first visit, two departed from the area during the study and the others refused sampling repeatedly). The number of subjects recruited in each DCC ranged from 4 to 29. The median number of days after 1st vaccine dose at V2 sampling was 7 for both groups (range 5-26) and, for V3, 28 (27-43) and after the 2nd were (V4) 7 (6-16) and (V5) 28 (21-48). The control (late vaccine) group had a higher mean number of siblings, but otherwise the groups were similar following randomization (table 1). There was no evidence to suggest a difference in antibiotic use between the groups at baseline (3/70 in vaccine group and 1/72 in controls), V2 (1/66 and 2/69) or V3 (3/65 and 3/72, respectively).

Virology results for the whole study period

Respiratory viral nucleic acid was detected ($CT \leq 35$) in 216/670 samples (from 144 children) among which 37 samples had more than one virus. Rhinovirus was the most frequently detected (16.8%), followed by coronavirus (4.4%), parainfluenza virus (3.3%), bocavirus (3.1%), enterovirus (3.1%) and respiratory syncytial virus (2.8%). Adenovirus, human metapneumovirus and parechovirus were found in fewer than 2.5% of samples. Influenza virus was found in 15 (2.3%) of which five were influenza A and ten were detected seven days after vaccination and so likely to represent vaccine virus (none of these children reported influenza-like-illness). We found no evidence for a difference in non-influenza viral detection rates between groups at baseline (table 1) or at visits 2 and 3 (22/66 and 24/64, $p=0.859$; 21/65 and 15/72, $p=0.130$, respectively). Rates of detection of rhinovirus, the most frequently detected virus, were also similar in both groups.

Bacterial prevalence visits 1-3

At baseline, carriage prevalence was high for *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* (68.3%, 77.6% and 57.8% respectively) and lower for *S. aureus* (10.6%); there was no evidence to suggest a difference between the groups (table 2). At V2 and V3 (7 and 28 days post early vaccination) there was no evidence to suggest a difference between the two study groups in the detection of these bacterial species apart from an apparent modest and transient increase in *H. influenzae* prevalence seen at V2. 46/66 (69.7%) were positive among the early vaccinees and 36/69 (52.2%) among the controls ($p=0.042$, adjusted for clustering by nursery).

Bacterial density visit 1-3

Changes in bacterial density were investigated in those children who were positive for carriage at baseline (V1). Amongst these children, there was no evidence to suggest a difference in mean *S. pneumoniae* density (GC/ml) between the study groups at baseline (V1) or seven days (V2) after the intervention. However, at V3 (28 days after vaccination) a six-fold higher mean bacterial density was found in children positive for carriage in the vaccinated group, whose values had risen compared to controls, whose values appeared to have fallen slightly over this period (table 3). There was no evidence of a difference at either V2 or V3 (7 or 28 days after vaccination) for *M. catarrhalis* or *H. influenzae*; there were too few positive children for *S. aureus* at baseline to permit statistical analysis (n=15) although there was a trend towards higher density carriage in vaccinees (online supplement figure E1).

Carriage over the whole study period

At the first visit, 45 children tested negative for carriage of *S. pneumoniae* (samples at visit 1 were unavailable for 9 children). Over the course of the study 42 of these subsequently tested positive, including 9 children before receiving a vaccine, 20 after receiving 1 dose and 10 after receiving 2 doses with the three remaining children leaving the study. Similarly to the findings of the between groups analysis, in the pre/post vaccine analysis across all visits there was little to suggest any differences in carriage prevalence after vaccination (one or two doses of LAIV) for *S. pneumoniae*, *M. catarrhalis* or *S. aureus* (table 4). However, once again, there was evidence for an effect of vaccine to increase *H. influenzae* prevalence, with higher odds of carriage after both one and two doses of LAIV, after controlling for confounding and allowing for clustering by nursery and repeated measures on children over time. Analyses of bacterial density were again restricted to those children with detectable carriage at baseline (in order to

assess changes in density as opposed to acquisition). Again, there was evidence of an increase in *S. pneumoniae* density with increasing doses of vaccine, in particular a 2.5 fold increase in density was observed after 2 vaccine doses, compared to pre-vaccine levels, when considering samples over all time points in the study (table 5). There was also evidence of vaccine-associated increases in *M. catarrhalis* density in carriers with a more than doubling observed after the first vaccine dose. Although point estimates post-vaccine were also raised relative to pre-vaccine values for *H. influenzae*, the confidence intervals were wide. Again too few children were carriers of *S. aureus* to permit analysis of density by this approach. The relationship between nasal discharge scores (SNOT score) and bacterial density was investigated by including nasal discharge scores in the *S. pneumoniae* model and evidence was found for an independent positive association with pneumococcal density (online supplement table E3).

Discussion

This is the first study to explore the effects of experimental respiratory viral infection upon bacterial upper respiratory colonization in humans. We show that infections with attenuated viruses can result in increases in carriage density of common bacteria or increases in acquisition, raising the possibility that wild type influenza and other viruses may have more marked such effects. Children carrying more bacteria are probably more infectious than those with fewer and it makes biological sense that such bacteria should evolve to sense viral rhinitis or the host response to it and exploit it as a transmission opportunity. In addition to proliferating, bacteria might change phenotype to one better suited to successful transit. Our findings also suggest that LAIV could be used as an experimental tool with which to elucidate relationships between nasal bacterial abundance and transmission rates between children.

Since the effectiveness of pneumococcal conjugate vaccines depends upon their impact on bacterial transmission both within and beyond the pediatric population, there are good practical reasons why the biology of this process should be elucidated in detail.

Does this study raise safety concerns about LAIV? We did not detect any safety signals associated with increased bacterial colonization density but the study was not powered to do so. Upper respiratory carriage of bacteria is not an illness and many authors assume it to be asymptomatic(22, 23). We have reported an association between carriage of *H. influenzae* and rhinitis in healthy children(4) and here we demonstrate an association for *S. pneumoniae* density independent of detectable viral nucleic acid and age. However, these symptoms are mild and the vaccine has been studied in clinical trials(10-13) and widely used in children aged 2 years and older without any safety signals which one might attribute to higher bacterial density such as otitis media, pneumonia or bacteremia and overall the vaccine protects against the former(10). One might expect evolutionary pressures to select for bacteria which exploit the ubiquitous respiratory viral infections of early childhood to promote colonization and transmission while minimizing serious pathology in their hosts.

An apparent short term effect of LAIV on carriage prevalence of *H. influenzae* in the randomized comparison was confirmed in multivariable analysis where it was also observed for the second vaccine dose. However measurement of colonization rates with this and other bacterial species was not the primary study aim and absolute numbers of carriers constituting the difference were small, so it is possible that this finding may have occurred by chance. If it is a genuine increase in bacterial acquisition induced by the vaccine, it has echoes of the observation of pneumococcal acquisition in ferrets experimentally infected with influenza (2).

In a recent adult pneumococcal challenge study, colonization was more likely in the presence of a respiratory virus(24) although this was not associated with higher carriage density, possibly reflecting differences between adults and children. Confirmatory studies of acquisition of *H. influenzae* and other bacterial species are needed. A twofold rise in *M. catarrhalis* density was seen in the comparison of pre- and post-vaccine doses but not in the randomized comparison most likely due to a population average effect (specifically, the big impact of those with very high colonization densities). We also found that colonization at the time of vaccination is associated with greater overall rises in density, suggesting that increases in bacterial load were not due to acquisition of new strains but proliferation of those already present (data not shown). This could be confirmed in future studies. We also found no effects of number of siblings (table 1) on the observed differences between groups (data not shown). There were too few *S. aureus* carriers to analyze the effects of the vaccine on density, although we saw apparently similar rises in density at V3 (28 days) those seen for *S. pneumoniae* (online supplement, figure E1) observations concordant with those made in animal models(15).

More generally, the use of randomization and the blinding of the laboratory analysis make it unlikely that our results were subject to bias or confounding and the size of the effects seen were sufficiently large to allow for possible biological effects on transmission rates. Although several bacterial species were studied, increasing the possibility of apparent differences arising by chance, individual upwards trends were consistently seen while secondary larger non-randomized but conservative multivariable analysis re-confirmed the positive findings of the randomized comparisons and extended them to other species when potential confounding factors, in particular age and clustering by nurseries, were taken into account.

The stepped wedge design used reduced the time period over which randomized comparisons could be made but allowed more children and samples to be included in a confirmatory pre-post-vaccine multivariate analysis and had the ethical and practical advantage of offering all participants protection against influenza. The higher drop-out rate in the early vaccine group was mostly evident after the third visit (figure 1) and thus did not influence the findings of the between group analysis (V1-3). That no placebo was used is a limitation but an administration device identical to the vaccine could not be obtained. Less than 30% of the available parents consented to participate which is high for a pediatric vaccine study, but raises the possibility of selection bias. We could not collect information on parents who refused or their children and so cannot exclude this possibility but have no reason to believe our subjects were unrepresentative of the population.

Our estimate of the effect of LAIV on bacterial carriage may be conservative. Available resources permitted us to conduct an individually randomized study. Through exposure to vaccinated children with elevated rates or density of colonization, controls may have also had their values elevated diluting the observed effect of the intervention. Future confirmatory studies would preferably have a cluster randomized design.

We detected and quantified species-specific bacterial DNA using PCR rather than viable bacteria by conventional culture techniques and thus will, in part, have been detecting DNA from non-viable organisms. Had cultures been done, doubtless some PCR-positive culture-negative individuals would have been found. However, we have recently shown that individuals with discordant PCR and culture results tend to have low density colonization(16). Although DNA may be present in the absence of living organisms, it nevertheless represents a footprint of recent bacterial presence and that is what this study was designed to measure.

Nevertheless it will be important to perform follow up confirmatory studies and to elucidate how the bacterial density measurements we report here relate to efficiency of transmission between colonized children.

Based on murine studies(15) our expectation was that changes in nasal bacterial colonization density would be evident around seven days after immunization and our observations of increases at 28 days were unexpected raising questions about the time course of this effect in children. As no intermediate samples between 7 and 28 days were taken, nor any more than 28 days after a vaccine dose, the timing of the peak of this effect remains unclear. Since bacteria can divide very rapidly, one can speculate that the time lag that rendered the density rise undetectable at 7 days, by when vaccine viral infection would have been well established, indicates that bacterial proliferation is triggered by the host response rather than the viruses themselves. It is also possible that the usually asymptomatic infections caused by attenuated cold-adapted vaccine viral strains induce relatively slow, small bacterial responses that were hard to detect at the earlier time point. Future studies should include additional sampling times to clarify the duration of this effect and the timing of its peak.

Although we present results on several bacterial species which commonly colonize children, we offer no information about serotypes of the studied species or the many others which are commonly present (25). We are now undertaking further analysis of these samples using microbiomic techniques to further explore viral-bacterial interactions.

In conclusion, we show that viral infections may induce species-specific changes both in bacterial density and acquisition and that it is important to study bacterial colonization as a continuous variable. Although not associated with disease, these effects may be important at

the population level especially in the context of more virulent wild-type respiratory virus infections which may have potent effects on bacterial transmission between individuals. Nearly all childhood vaccination programs rely to an extent upon the interruption of transmission of infections, so our findings should encourage further human studies of transmission dynamics.

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Declaration of interests

VT has received travel grants from GSK to attend international conferences. AF has received research grants from GSK, AstraZeneca, Alios, Pfizer, Novartis and SPSMD and consultancy fees and speaking honoraria, all paid to his employers, from the same companies as well as Takeda. He is a member of the WHO European Technical Advisory Group of Experts on Immunisation and the UK Department of Health's Joint Committee on Vaccination and Immunisation and subcommittees. HC has received a grant, paid to her employer, from AstraZeneca, outside the submitted work. BMA, IV and PM have no declarations of interests.

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Figure legends

Figure 1. Study Consort flow diagram

Tables

Table 1. Clinical characteristics of the two study groups at randomization

	Vaccine n=74*	Controls n=77	p
Age (months)	38.3 (7.8)	40.3 (6.7)	0.11
Male	35/74 (47.3)	37/77 (48.1)	0.93
Mean number of siblings	0.69 (0.77)	1.04 (1.02)	0.02
Underlying medical condition	11/74 (14.9)	17/77 (22.1)	0.25
Parental smoking	6/73 (8.2)	5/74 (6.8)	0.74
National vaccination schedule not complete	2/73 (2.7)	1/77 (1.3)	0.53
Given influenza vaccine - previous 2 seasons	4/73 (5.5)	3/75 (4.0)	0.67
Non-influenza viral nucleic acid detected	24/69	21/71	0.512

*Values are mean (standard deviation) or n/N (percentage)

Table 2. Carriage prevalence of bacterial species in early vaccine and control groups by visit

	<i>S. pneumoniae</i>				<i>M. catarrhalis</i>				<i>H. influenzae</i>				<i>S. aureus</i>			
	<i>Children with carriage n/N (%)</i>		OR (95% CI)*	p	<i>Children with carriage n/N (%)</i>		OR (95% CI)	p	<i>Children with carriage n/N (%)</i>		OR (95% CI)	p	<i>Children with carriage n/N (%)</i>		OR (95% CI)	p
	Controls	Vaccine			Controls	Vaccine			Controls	Vaccine			Controls	Vaccine		
Visit 1 (baseline)	51/72 (70.8)	46/70 (65.7)	0.79 (0.39 to 1.60)	0.512	52/66 (78.8)	52/68 (76.5)	0.90 (0.39 to 2.08)	0.810	39/72 (54.2)	43/70 (61.4)	1.36 (0.69 to 2.66)	0.376	8/72 (11.1)	7/70 (10.0)	0.89 (0.30 to 2.63)	0.836
Visit 2 (+/-7 days)	48/69 (69.6)	45/66 (68.2)	0.94 (0.45 to 1.94)	0.862	56/66 (84.9)	54/66 (81.8)	0.80 (0.32 to 2.01)	0.641	36/69 (52.2)	46/66 (69.7)	2.13 (1.03 to 4.44)	0.042	5/69 (7.3)	4/66 (6.1)	0.83 (0.21 to 3.30)	0.792
Visit 3 (+/-28 days)	52/72 (72.2)	41/65 (63.1)	0.66 (0.32 to 1.36)	0.255	52/68 (76.5)	50/65 (76.9)	1.01 (0.45 to 2.28)	0.983	47/72 (65.3)	44/64 (68.8)	1.19 (0.57 to 2.48)	0.639	5/72 (6.9)	7/65 (10.8)	1.62 (0.49 to 5.37)	0.433

*OR of carriage at each visit in early vaccine group compared to control group with nursery (random effect, intercept) in the model.

Table 3. Carriage density of bacterial species in early vaccine and control groups, in individuals who were carriage positive at baseline

	<i>S. pneumoniae</i>				<i>M. catarrhalis</i>				<i>H. influenzae</i>			
	<i>Mean carriage*</i>		Mean ratio**	p	<i>Mean carriage</i>		Mean ratio	p	<i>Mean carriage</i>		Mean ratio	p
	<i>density GC/ml (SD)</i>	<i>density GC/ml (SD)</i>			<i>density GC/ml (SD)</i>	<i>density GC/ml (SD)</i>			<i>density GC/ml (SD)</i>	<i>density GC/ml (SD)</i>		
	Controls	Vaccine	(95% CI)		Controls	Vaccine	(95% CI)		Controls	Vaccine	(95% CI)	
Visit 1 (baseline)	3297 (6394)	2687 (4635)	0.78 (0.38 to 1.61)	0.501	15399 (58725)	13673 (40268)	0.91 (0.38 to 2.19)	0.830	17286 (38941)	18379 (41310)	1.11 (0.39 to 3.20)	0.846
Visit 2 (7 days)	3484 (9640)	4202 (8481)	1.81 (0.81 to 4.04)	0.146	19589 (90043)	19871 (42525)	1.82 (0.78 to 4.27)	0.165	10505 (19159)	29985 (73937)	2.19 (0.76 to 6.30)	0.145
Visit 3 (28 days)	1935 (5275)	16687 (56920)	6.35 (2.30 to 17.61)	<0.001	25597 (83640)	18640 (42995)	0.92 (0.38 to 2.20)	0.845	26858 (96327)	24550 (46652)	2.15 (0.73 to 6.34)	0.167

*Mean carriage density in those who were positive. SD: standard deviation. **Arithmetic mean ratio. Estimates of arithmetic mean ratio allows for clustering by nursery (random effect, intercept) and so is not the simple quotient of the respective means. Too few children carried *S. aureus* for statistical analysis.

Table 4. Odds ratios (generalized linear mixed models) for carriage prevalence of bacterial species after vaccination including all visits in modified intention-to-treat population

	<i>S. pneumoniae</i>		<i>M. catarrhalis</i>		<i>H. influenzae</i>		<i>S. aureus</i>	
	OR (95% CI)*	p	OR (95% CI)*	P	OR (95% CI)*	p	OR (95% CI)*	p
Unvaccinated	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
1 LAIV dose	0.84 (0.52 to 1.36)	0.777	1.15 (0.62 to 2.15)	0.091	2.23 (1.29 to 4.21)	0.015	0.81 (0.34 to 1.90)	0.868
2 LAIV dose	0.90 (0.44 to 1.81)		2.59 (0.94 to 7.12)		3.46 (1.33 to 9.02)		0.73 (0.20 to 2.65)	

*OR of carriage adjusted by months of age, gender, presence of smoker in the home, number of siblings, presence of viral nucleic acid, time (week in the study) with nursery and subject ID as random effects (intercept).

Data for the 2nd LAIV dose relate only to the group who received vaccine at visits 1 and 3.

Table 5. Arithmetic mean ratio (generalized linear mixed models) for carriage density of bacterial species after vaccination including all visits in modified intention-to-treat population

	<i>S. pneumoniae</i>		<i>M. catarrhalis</i>		<i>H. influenzae</i>	
	Arithmetic mean ratio (95% CI)*	p	Arithmetic mean ratio (95% CI)*	p	Arithmetic mean ratio (95% CI)*	p
Unvaccinated	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
1 LAIV dose	1.24 (0.69 to 2.23)	0.064	2.21 (1.22 to 4.00)	0.029	1.89 (0.98 to 3.65)	0.161
2 LAIV dose	2.68 (1.11 to 6.47)		1.99 (0.84 to 4.73)		1.69 (0.69 to 4.17)	

* Arithmetic mean ratio (95% CI) of carriage density adjusted by months of age, gender, smoker in the home, number of siblings, presence of viral nucleic acid, time (week in the study) with nursery and subject ID as random effects (intercept). Too few children carried *S. aureus* for statistical analysis. Data for the 2nd LAIV dose relate only to the group who received vaccine at visits 1 and 3.

Figures

Figure 1. Study Consort flow diagram

